

## AN UNEXPECTED LIFE IN RESEARCH

*Julius Axelrod*

Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, Maryland 20892

### BEGINNINGS

Successful scientists are generally recognized at a young age. They go to the best schools on scholarships, receive their postdoctoral training fellowships at prestigious laboratories, and publish early. None of this happened to me.

My parents emigrated at the beginning of this century from Polish Galicia. They met and married in America, where they settled in the Lower East Side of New York, then a Jewish ghetto. My father, Isadore, was a basketmaker who sold flower baskets to merchants and grocers. I was born in 1912 in a tenement on East Houston Street in Manhattan.

I attended PS22, a school built before the Civil War. Another student at that school before my time was I. I. Rabi, who later became a world-renowned physicist. After PS22 I attended Seward Park High School. I really wanted to go to Stuyvesant, a high school for bright students, but my grades were not good enough. Seward Park High School had many famous graduates, mostly entertainers: Zero Mostel, Walter Mathau, and Tony Curtis. My real education was obtained at the Hamilton Fish Park Library, a block from my home. I was a voracious reader and read through several books a week—from Upton Sinclair, H. L. Menken, and Tolstoy to pulp novels such as the Frank Merriwell and Nick Carter series.

After graduating from Seward Park High School, I attended New York University in the hope that it would give me a better chance to get into medical school. After a year my money ran out, and I transferred to the tuition-free City College of New York in 1930. City College was a proletarian Harvard, which subsequently graduated seven Nobel Laureates. I majored in biology and chemistry, but my best grades were in history, philosophy, and literature. Because I had to work after school, I did most of my studying

during the subway trip to and from uptown City College. Studying in a crowded, noisy New York subway gave me considerable powers of concentration. When I graduated from City College, I applied to several medical schools but was not accepted by any.

In 1933, the year I graduated from college, the country was in the depths of a depression. More than 20% of the working population was unemployed, and there were few jobs available for City College graduates. I had heard about a laboratory position that was available at the Harriman Research Laboratory at New York University, and although the position paid \$25 a month, I was happy to work in a laboratory. I assisted Dr. K. G. Falk, a biochemist, in his research on enzymes in malignant tumors. I also purified salts for the preparation of buffer solutions and determined their pH. The instrument used to measure pH at that time was a complex apparatus; the glass electrode occupied almost half a room. In 1935 the laboratory ran out of funds, and I was fortunate to get a position as a chemist in the Laboratory of Industrial Hygiene. This laboratory was a nonprofit organization and was set up by New York City's Department of Health to test vitamin supplements added to foods. I worked in the Laboratory of Industrial Hygiene from 1935 to 1946.

My duties there were to modify published methods for measuring vitamins A, B, B<sub>2</sub>, C, and D so that they could be assayed in various food products that city inspectors randomly collected. Vitamins had just been introduced at that time, and the New York City Department of Health wanted to establish that accurate amounts of vitamins were added to milk and other food products. The methods used for measuring vitamins then were chemical, biological, and microbiological. It required some ingenuity to modify the methods described in the literature to assays of food products. This experience in modifying methods was slightly more than routine, but it proved to be useful in my later research. The laboratory subscribed to the *Journal of Biological Chemistry*, which I read with great interest. Reading this journal made it possible to keep up with advances in the enzymology, nutrition, and methodology. During the time I was in the Laboratory of Industrial Hygiene, I received a MS degree in chemistry at New York University in 1942 by taking courses at night. My thesis was on the ester-hydrolyzing enzymes in tumor tissues. Because of the loss of one eye in a laboratory accident, I was deferred from the draft during World War II. In 1938, I married Sally Taub, a graduate of Hunter College who later became an elementary school teacher. We had two sons, Paul and Alfred, born in 1946 and 1949.

## FIRST EXPERIENCE IN RESEARCH: GOLDWATER MEMORIAL HOSPITAL

I expected that I would remain in the Laboratory of Industrial Hygiene for the rest of my working life. It was not a bad job, the work was moderately

interesting, and the salary was adequate. One day early in 1946 the Institute for the Study of Analgesic and Sedative Drugs approached the president of the Laboratory of Industrial Hygiene with a problem. The president of the Laboratory at that time was George B. Wallace, a distinguished pharmacologist who had just retired as Chairman of the Department of Pharmacology at New York University. Many analgesic preparations contained nonaspirin analgesics, such as acetanilide or phenacetin. Some people who became habituated to these preparations developed methemoglobinemia. The Institute for the Study of Analgesic and Sedative Drugs offered a small grant to the Laboratory of Industrial Hygiene to find out why acetanilide and phenacetin taken in large amounts produced methemoglobinemia. Dr. Wallace asked me if I would like to work on this problem. I had little experience in this kind of research, and he suggested that I consult Dr. Bernard "Steve" Brodie. Dr. Brodie was a former member of the Department of Pharmacology at New York University and was doing research at Goldwater Memorial Hospital, a New York University Division.

I met with Brodie in February 1946 to discuss the problem of analgesics. It was a fateful meeting for me. Brodie and I talked for several hours about what kind of experiments could be done to find out how acetanilide might produce methemoglobinemia. Talking to Brodie about research was one of my most stimulating experiences. He invited me to spend some time in his laboratory to work on this problem. One of a number of possible products of acetanilide that would cause the toxic effects was aniline. It had previously been shown that aniline could produce methemoglobinemia. Thus, one approach was to find out whether acetanilide could be deacetylated to form aniline in the body. With the help and guidance of Steve Brodie, I developed a method for measuring aniline in nanogram amounts in urine and plasma. After the administration of acetanilide to human subjects, aniline was found to be present in urine and plasma. A direct relationship between the level of aniline in blood and the amount of methemoglobin present was soon observed (1). This was my first taste of real research, and I loved it.

Very little acetanilide was found in the urine, suggesting extensive metabolism in the body. Since acetanilide was almost completely transformed in the body, we looked for other metabolic products. Methods to detect possible metabolites, *p*-aminophenol and *N*-acetyl-*p*-aminophenol, were developed that were specific and sensitive enough to be used in the plasma and urine. Within a few weeks, we identified the major metabolite as hydroxylated acetanilide *N*-acetyl-*p*-aminophenol and its conjugates. This metabolite was also found to be as potent as acetanilide in analgesic activity. By taking serial plasma samples, acetanilide was shown to be rapidly transformed to *N*-acetyl-*p*-aminophenol (1). After the administration of *N*-acetyl-*p*-aminophenol, negligible amounts of methemoglobin were produced. As a result of these studies, Brodie and I stated in our paper (1), "the results are

compatible with the assumption that acetanilide exerts its action mainly through N-acetyl-*p*-aminophenol [now known as acetaminophen]. The latter compound administered orally was not attended by the formation of methemoglobin. It is possible therefore, that it might have distinct advantages over acetanilide as an analgesic." This was my first paper, and I was determined to continue doing research.

Soon after Brodie and I examined the physiological disposition and metabolism of acetanilide, we turned our attention to a related analgesic drug, phenacetin (acetophenetidin). I spent some time developing sensitive and specific methods for the identification of phenacetin and its possible metabolite, *p*-phenetidine. Brodie and I soon found that in humans, the major metabolic product was also N-acetyl-*p*-aminophenol arising from the deethylation of the parent compound (2). A minor metabolite was *p*-phenetidine, which we found was responsible for the methemoglobinemia formed after the administration of large amounts of phenacetin to dogs. After the administration of phenacetin to human subjects, N-acetyl-*p*-aminophenol was rapidly formed. The speed and the amount with which N-acetyl-*p*-aminophenol was formed in the body suggested that the analgesic activity resided in its deethylated metabolite.

The laboratories at Goldwater Memorial Hospital where I began my research career were set up during World War II to test newly synthesized antimalarial drugs for their clinical effectiveness. Early in the war, the Japanese had cut off most of the world's supply of the antimalarial quinine. James Shannon, then a renal physiologist at New York University, was put in charge of this program. Shannon had the remarkable capacity to pick the right young people to carry out research in the antimalarial project. Members of the team that worked at Goldwater in addition to Steve Brodie were Sid Udenfriend, Robert Berliner, Bob Bowman, Tom Kennedy, and Gordon Zubrod. The atmosphere at Goldwater was highly stimulating, and an outpouring of important new findings resulted. It was in this atmosphere that, in a period of a few years, I became a researcher.

After completion of the studies on acetanilide and phenacetin, Brodie invited me to stay on at Goldwater to study the fate of other analgesic drugs. We received a small grant from the Institute for the Study of Analgesic and Sedative Drugs, and the Laboratory of Industrial Hygiene paid my salary. Another drug we investigated was the analgesic antipyrine. A sensitive method for the detection of this drug was developed, which has since been used by other investigators as a marker to determine the activity of drug-metabolizing enzymes in vivo. We identified 4-hydroxyantipyrine and its sulfate conjugate as metabolites of antipyrine. We also observed that antipyrine distributed in the same manner as body water. Because of this property, antipyrine has been used for the measurement of body water. Another

analgesic we studied was aminopyrine. We found that this drug was demethylated to aminopyrine and N-acetylated to N-acetylaminopyrine. Many of the drugs whose fate Brodie and I studied were later used by many investigators as substrates for the microsomal drug-metabolizing enzymes: aminopyrine for N-demethylation, phenacetin for O-dealkylation, and aniline for hydroxylation. Together with Jack Cooper, we developed a method for measuring the anticoagulant dicoumerol in plasma. In a study on the disposition of dicoumerol in humans, an exceedingly wide difference in the plasma levels of this drug was found, suggesting genetic differences in drug metabolism.

## MOVE TO THE NATIONAL HEART INSTITUTE

Because I did not have a doctoral degree, I realized that I would have little chance for advancement in any hospital attached to an academic institution. I had neither the inclination nor the money to spend several years getting a PhD, so I decided to join the National Heart Institute as a research chemist. In 1949, Shannon was chosen as the director of the newly organized National Heart Institute in Bethesda, and he offered me a position. Also coming to the National Institutes of Health (NIH) at that time were many members of the Goldwater staff—Brodie, Udenfriend, Berliner, Kennedy, and Bowman.

At the National Heart Institute from 1950 to 1952, I collaborated with Brodie and his staff on the metabolism of analgesics and adrenergic blocking agents and the actions of ascorbic acid on drug metabolism. After a while, I became dissatisfied with working with a large team and was allowed to work independently. The first problem I chose was an examination of the physiological disposition of caffeine in man. Very little was known about the physiological disposition and metabolism of this widely used compound. A method for measuring caffeine in biological material was developed, and the plasma half-life and distribution were determined (3). Because of my work on analgesics and caffeine, I was delighted to be elected without a doctorate as a member of the American Society of Pharmacology and Experimental Therapeutics in 1953. K. K. Chen and Steve Brodie were my sponsors.

At that time, I became intrigued with the sympathomimetic amines. In 1910, Barger and Dale reported that numerous  $\beta$ -phenylethanolamine derivatives simulated the effects of sympathetic nerve stimulation with varying degrees of intensity and precision, and they coined the term *sympathomimetic amines*. Sympathomimetic amines such as amphetamine, mescaline, and ephedrine also produced unusual behavioral effects. In 1952, very little information concerning the metabolism and physiological disposition of these amines was known. Because of my experience in drug metabolism, I decided

to undertake a study on the fate of ephedrine and amphetamine. In retrospect, this was an important decision.

The first amine that I studied was ephedrine. Ephedrine, the active principle of *Ma Huang*, an herb used by ancient Chinese physicians, was introduced to modern medicine by Chen and Schmidt in 1930. I soon found that ephedrine was transformed in animals by two pathways (demethylation and hydroxylation) to yield metabolic products that had pressor activity. Various animal species showed considerable differences in the relative importance of these two metabolic routes. The next sympathomimetic amines I examined were amphetamine and methylamphetamine. These compounds were shown to be metabolized by a variety of metabolic pathways including hydroxylation, demethylation, deamination, and conjugation. Marked species variations in the transformation of these drugs were also observed.

## THE DISCOVERY OF THE MICROSOMAL DRUG METABOLIZING ENZYMES

When amphetamine was given to rabbits, it disappeared without a trace. This puzzled me, so I decided to look for enzymes that metabolized this drug. I had no experience in enzymology, but there were many outstanding enzymologists in Building 3 on the NIH campus where my laboratory was located. Gordon Tomkins, who occupied the lab bench next to mine, offered me good advice. Gordon had the capacity for demystifying enzymology and told me that all I needed to start in vitro experiments was a method for measuring amphetamine, an animal liver, and a razor blade. I did my first in vitro experiment with rabbit liver in January 1953. When rabbit liver slices were incubated in Krebs Ringer-buffer solutions with amphetamine, the drug was almost completely metabolized. Upon homogenization of the rabbit liver, amphetamine was not metabolized unless cofactors such as DPN (NAD), TPN (NADP), and ATP were added. I then decided to examine which subcellular fraction was responsible for transforming amphetamine. Hogeboom and Schneider had just described a reproducible method for separating the various subcellular fractions by homogenizing tissue in isotonic sucrose and subjecting the homogenate to differential centrifugation. After separation of nuclei, mitochondria, microsomes, and the cytosol, none of these fractions were able to metabolize amphetamine, even in the presence of added cofactors. However, when the microsomes and cytosol were combined, amphetamine rapidly disappeared upon the addition of DPN, TPN, and ATP. At that time Bert La Du, a colleague at the NIH, observed that the demethylation of aminopyrine in a dialyzed rat liver whole homogenate required TPN. In a subsequent experiment I found that amphetamines were metabolized in a dialyzed preparation of microsomes and cytosol in the presence of TPN, but

not DPN or ATP. However, when the microsomes and cytosol were separately incubated, little or no drug was metabolized, despite the addition of TPN. I realized then that I was dealing with a unique enzymatic reaction.

Before I went further, I decided to identify the metabolic products of amphetamine produced when the combined microsomes and cytosolic fraction were incubated with TPN. One of the possible metabolic pathways might be deamination, leading to the formation of phenylacetone. After incubation of amphetamine with the above preparations, phenylacetone and ammonia were identified. These results indicated that amphetamine was deaminated by an oxidative enzyme requiring TPN either in the microsomes or cytosol to form phenylacetone and ammonia. Because of its properties and the structure of the substrate, it was apparent that this enzyme differed from another deaminating enzyme, monoamine oxidase.

Where was the enzyme located, in the microsomes or the soluble supernatant fraction? An approach that I used to locate the enzyme was to heat each fraction for a few minutes at 55°C, a temperature that would destroy heat-sensitive enzymes. When the cytosol was heated to 55°C and then added to unheated microsomes and TPN, amphetamine was deaminated. When the microsomes were heated and added to the cytosol fraction together with TPN, amphetamine was not metabolized. This was a crucial experiment, which demonstrated that a heat-labile enzyme that deaminated amphetamines was localized in the microsomes and that the cytosol provided factors involving TPN necessary for this reaction.

Bernard Horecker, then working in Building 3, prepared several substrates for the TPN-requiring dehydrogenase for his classic work on the pentose phosphate pathway. He generously supplied me with these substrates, which I could test on my preparations. I found that the addition of glucose-6-phosphate, isocitric acid, or phosphogluconate acid, together with TPN, to unwashed microsomes transformed amphetamines. A reaction common to these substrates is the generation of TPNH, suggesting that the enzymes in the cytosol fraction were reducing TPN. Incubating microsomes with a TPNH-generating system using glucose-6-phosphate and glucose-6-phosphate dehydrogenase resulted in the deamination of amphetamines. Upon incubation of chemically synthesized TPNH, microsomes, and oxygen, amphetamine was deaminated. At about the same time, I also found that ephedrine was demethylated to norephedrine and formaldehyde by enzymes present in rabbit microsomes that required TPNH and oxygen. By the end of June 1953, I felt confident that I had described a new enzyme that was localized in the microsomes, required TPNH and oxygen, and could deaminate and demethylate drugs. I reported these findings at the 1953 fall meeting of the American Society of Pharmacology and Experimental Therapeutics (4, 5).

After the description of the TPNH-requiring microsomal enzymes that

deaminated amphetamine and demethylated ephedrine, several members of the Laboratory of Chemical Pharmacology at the NIH described similar enzyme systems that could metabolize other drugs by a variety of pathways, N-demethylation of aminopyrine (La Du, Gaudette, Trousof, and Brodie), oxidation of barbiturates (Cooper and Brodie), and the hydroxylation of aniline (Mitoma and Udenfriend) (6). In a study of the N-demethylation of narcotic drugs that I made soon after, it became apparent that there were multiple microsomal enzymes that required TPNH and  $O_2$  (7). Research on the microsomal enzymes (now called cytochrome-P450 monooxygenases) has expanded enormously and has had a profound influence in biomedical sciences, ranging from studies of metabolism of normally occurring compounds to carcinogenesis. In retrospect, the discovery of the microsomal enzymes is among the best work I did.

Brodie and I were struck by the findings of investigators at Smith Kline & French that SKF525A, a compound with little pharmacological action of its own, prolonged the duration of action of a wide variety of drugs. We conjectured that the compound might exert its effects by inhibiting the metabolism of drugs. The effects of SKF525A on the metabolism of ephedrine in dogs and on the metabolism and duration of action of hexabarbital were examined. We found that SKF525A slowed the demethylation of ephedrine in the intact dog. It also prolonged the presence of hexabarbital in the plasma and the sleeping time in rats and dogs. Thus, the ability of SKF525A to prolong the action of drugs could be explained by its ability to slow their metabolism. As soon as the microsomal enzymes were described, it was observed that SKF525A inhibited this class of enzymes. Subsequently, SKF525A was widely used as an inhibitor of the microsomal enzymes.

The effect of the microsomal enzymes on the duration of drug actions was examined with the collaboration of Gertrude Quinn, a graduate student at George Washington University, and Steve Brodie. Since sleeping time of hexabarbital was easy to measure, we chose that drug to make this study. Cooper and Brodie had found that hexabarbital was metabolized by microsomal enzymes in the liver (6). The sleeping time of a given dose of hexabarbital was compared with its plasma half-life and with the activity of a liver enzyme preparation using the barbiturate as a substrate in a number of mammalian species. There were considerable differences in the plasma half-life, sleeping time, and enzyme activity among the various species (8). A high correlation was observed between the plasma half-life and sleeping time of the barbiturate. There was also an inverse relationship between the duration of action of hexabarbital and its ability to be metabolized by the microsomal enzymes.

In 1956, I reported that narcotic drugs such as morphine, meperidine, and methadone were N-demethylated by the liver microsomes requiring TPNH

and  $O_2$  (7). Differences in the rate of N-demethylation of various narcotic drugs in several species made it apparent more than one enzyme was involved in their N-demethylation. There was also a marked sex difference in the N-demethylation of narcotic drugs by rat liver microsome enzymes. Microsomes obtained from male rats were found to N-demethylate narcotic drugs much faster than those from female rats. When testosterone was administered to oophorectomized female rats, the activity of the demethylating enzyme was markedly increased. Estradiol given to male rats decreased the enzyme activity. Subsequent work by many investigators found similar sex differences in microsomal enzyme activity for many metabolic pathways.

While working on the metabolism of narcotic drugs, I observed that the repeated administration of narcotic drugs not only produced tolerance to these drugs, but also markedly reduced the ability to N-demethylate them enzymatically (9). There was also a correlation between the rate of demethylation of opiate substrates and their cross-tolerance to morphine. Opiate antagonists not only blocked the development of tolerance, but also prevented the reduction of enzyme activity. On the basis of these observations, a mechanism for tolerance to narcotic drugs was proposed. In a paper reporting these experiments, the following statement was made: "The changes in enzyme activity in morphine-treated rats suggests a mechanism for the development of tolerance to narcotic drugs, if one assumes that enzymes which N-demethylate narcotic drugs and the receptors for these drugs are probably closely related. The continuous interaction of narcotic drugs with the demethylating enzymes inactivates the enzymes. Likewise the continuous interaction of narcotic drugs with their receptors may inactivate the receptors. Thus, a decreased response to narcotic drugs may develop as a result of unavailability of receptor sites." This hypothesis stimulated considerable critical reaction, mostly negative.

Although I had just described the physiological disposition of caffeine, demonstrated the variety of metabolic pathways of amphetamine and ephedrine, and independently described the microsomal enzymes and their role in drug metabolism, it was difficult for me to obtain a promotion to a higher rank at the National Heart Institute because I had no doctorate. I decided to get a PhD degree at George Washington University, since few courses were required if a candidate already had an MS degree. However, it would be necessary to take demanding comprehensive examinations in several subjects. Paul K. Smith, then Chairman of Pharmacology, accepted me as a graduate student in his department. He allowed me to submit my work on the metabolism of sympathomimetic amines and the microsomal enzyme for my dissertation. I took a year off to attend courses at George Washington University, and I found going back to school pleasant and challenging. A few of the medical students did better than I did in the pharmacology examinations. On one

occasion a multiple-choice question on antipyrine, a compound on which I published several papers, was asked, and I gave the wrong answer. After a year's study, I passed a tough comprehensive examination, and my thesis *The fate of phenylisopropylamines* was accepted. In 1955, at the age of 42 years, I received my PhD.

## SETTING UP A LABORATORY AT THE NATIONAL INSTITUTE OF MENTAL HEALTH

While studying for my PhD, I was invited by Edward Evarts to set up a Section of Pharmacology in his Laboratory of Clinical Sciences at the National Institute of Mental Health (NIMH). To get started on my new position at the NIMH I took a few afternoons off my classes at George Washington University to do laboratory work. I thought that a study of the metabolism and distribution of LSD would be an appropriate problem for my new laboratory in the NIMH. LSD was then used as an experimental drug by psychiatrists to study abnormal behavior. Bob Bowman at the NIH was in the process of building a spectrofluorometer. He was kind enough to let me use his experimental model, which allowed me to develop a very sensitive fluorometric assay for LSD. This made it possible to measure the nanogram amounts found in brain and other tissues. This instrument later became the well-known Aminco Bowman spectrofluorometer. The availability of this instrument made it possible for many laboratories to devise sensitive methods for the measurement of endogenous epinephrine, norepinephrine, dopamine, and serotonin in brain and other tissues. These newly developed methods for biogenic amines were crucial in the subsequent rapid expansion in neurotransmitter research.

Just before I left the Heart Institute, I read a report in the literature that uridine diphosphate glucuronic acid (UDPGA) was a necessary cofactor for the formation of phenolic glucuronides in a cell-free preparation of livers. Jack Strommiger, a biochemist then at the NIH, and I discussed the possible mechanism for the enzymatic synthesis of UPDGA. We suspected that it would arise from the oxidation of uridine diphosphate glucose (UDPG) by either TPN or DPN. We obtained a sample of UDPG from Herman Kalckar and did a preliminary experiment in which I measured the disappearance of morphine in guinea pig liver. When morphine was incubated with guinea pig liver microsomes and soluble fraction with DPN and UDPG, morphine was metabolized; TPN had no effect. When either DPN, UDPG, soluble fraction, or liver was omitted, the disappearance of morphine was negligible. After a period of incubation during which the mixture was heated in 1N HCl, the morphine that disappeared was recovered. These experiments suggested that morphine was enzymatically conjugated in the presence of UDPG and DPN,

presumably by the formation of UDPGA followed by morphine glucuronide. I had little time to continue this problem because I was in the process of getting my PhD. Strommiger and coworkers then went on to purify an enzyme UDPG dehydrogenase that formed UPDGA from UDPG and DPN.

After completion of my PhD, I returned to the glucuronide problem in my new laboratory at the NIMH. As expected from my preliminary experiment with morphine, I found that morphine and other narcotic drugs formed glucuronide conjugates by an enzyme present in liver microsomes that required UDPGA. Working together, Joe Inscoc, a graduate student at George Washington University, and I showed that glucuronide formation could be induced by benzpyrene and 3-methylcholanthrene.

The work on glucuronide conjugation led to a study on the role of glucuronic acid conjugation on bilirubin metabolism. Rudi Schmid, then at the NIH, made the interesting observation that bilirubin was transformed to a glucuronide. Schmid and I then went on to describe the enzymatic formation of bilirubin glucuronide by enzymes in the liver requiring UDPGA. This conjugating enzyme served as a mechanism for inactivating bilirubin. This led to an interesting clinical observation concerning a defect in glucuronide formation. In congenital jaundice there is a marked elevation of free bilirubin in the blood. This suggested to us that something might be wrong with glucuronide formation in this disease. The availability of a mutant strain of rats (Gunn rats) that exhibited congenital jaundice made it possible to examine whether the glucuronide-forming enzyme was defective. We then went on to demonstrate that these rats showed a marked defect in the ability to synthesize glucuronides from UDPGA (10). Glucuronide formation was also examined in humans with congenital jaundice by measuring the rate and magnitude of plasma acetaminophen glucuronide after the administration of the acetaminophen. A defect in glucuronide formation in this disease was demonstrated.

## CATECHOLAMINE RESEARCH

When I joined the NIMH, I knew very little about neuroscience. My impression of neuroscience then was that it was mainly concerned with electrophysiology, brain anatomy, and behavior. These subjects were to me somewhat strange and esoteric and concerned with complicated electronic equipment. I believed that an investigator had to be a gifted experimentalist and theorist to do research in the neurosciences. Ed Evarts, my lab chief, assured me that I could work on whatever problem I thought would be likely to yield new information. The philosophy of Seymour Kety, then head of the Intramural Programs of the NIMH, was to allow investigators working in the laboratories of the NIMH to do their research on whatever was potentially productive and important. Kety believed that without sufficient basic knowl-

edge about the life processes, doing targeted research on mental illness would be a waste of time and money.

Instead of working on a neurobiological problem, I thought it would be best to work on one that I knew something about, and that might be appropriate to the mission of the NIMH. I began to experiment on the metabolism and physiological disposition of LSD and the enzymes involved in the metabolism of narcotic drugs. I also worked on the enzymatic synthesis of glucuronides described above.

Although the NIMH administrators were supportive of the type of research I was doing, I still felt guilty that I was not working on some aspect of the nervous system or mental illness. Dr. Kety, in a seminar to our laboratory, gave a fascinating account of the findings of two Canadian psychiatrists. They reported that adrenochrome produced schizophreniclike hallucinations when it was ingested. Because of these behavioral effects, they proposed that schizophrenia could be caused by an abnormal metabolism of epinephrine to adrenochrome. I was intrigued by this proposal. In searching the literature, I was surprised to find that little was known about the metabolism of epinephrine at that time, in 1957. In view of the provocative hypothesis about the abnormal metabolism of epinephrine in schizophrenia, I decided to work on the metabolism of epinephrine. Epinephrine was then believed to be metabolized and inactivated by deamination by monoamine oxidase. However, with the introduction of monoamine oxidase inhibitors by Albert Zeller and coworkers, it was observed that, after the inhibition of monoamine oxidase in vivo, the physiological actions of administered epinephrine were still rapidly ended. This indicated that enzymes other than monoamine oxidase metabolized epinephrine. A possible route of metabolism of epinephrine might be via oxidation. I spent several months looking for oxidative enzymes for epinephrine without any success.

An abstract in the March 1957 *Federation Proceedings* gave me an important clue regarding a possible pathway for the metabolism of epinephrine. In this abstract, Armstrong and coworkers reported that patients with norepinephrine-forming tumors (pheochromocytomas) excreted large amounts of an O-methylated product, 3-methoxy-4-hydroxymandelic acid (VMA) (11). This suggested that this metabolite could be formed by the O-methylation and deamination of epinephrine or norepinephrine. The O-methylation of catecholamines was an intriguing possibility that could be experimentally tested. A potential methyl donor could be S-adenosylmethionine. That afternoon I incubated epinephrine with a homogenate of rat liver, ATP, and methionine. I did not have S-adenosylmethionine available, but Cantoni had shown that an enzyme in the liver could convert ATP and methionine to S-adenosylmethionine (12). I found that epinephrine was rapidly metabolized in the presence of ATP, methionine, and liver homogenate.

When either ATP or methionine was omitted or the homogenate was heated, there was a negligible disappearance of epinephrine. This experiment suggested that epinephrine was O-methylated in the presence of a methyl donor, presumably S-adenosylmethionine. In a following experiment, I obtained S-adenosylmethionine and observed that incubating liver homogenate with the methyl donor resulted in the metabolism of epinephrine. The most likely site of methylation would be on the *meta* hydroxyl group of epinephrine to form 3-O-methylepinephrine. I prevailed on my colleague Bernhard Witkop, a bioorganic chemist, to synthesize the O-methyl metabolite of epinephrine. A few days later Sero Senoh, a visiting scientist in Witkop's laboratory, synthesized meta-O-methylepinephrine, which we named metanephrine. After incubating liver and S-adenosylmethionine, the metabolite formed from epinephrine was identified as metanephrine, indicating the existence of an O-methylating enzyme. The O-methylating enzyme was purified and found to O-methylate catechols, including norepinephrine, dopamine, L-DOPA, and synthetic catechols, but not monophenols (13). In view of the substrate specificity, the enzyme was named catechol-O-methyltransferase (COMT). The enzyme was found to be widely distributed in tissues, including the brain.

Injecting catecholamines into animals resulted in the excretion of the respective O-methylated metabolites. We soon identified normally occurring O-methylmetabolites such as normetanephrine, metanephrine, 3-methoxy tyramine, and 3-methoxy-4-hydroxyphenylglycol (MHPG) in liver and brain. As a result of the discovery of the O-methylation metabolites, the pathways of catecholamine metabolism were clarified (13). Catecholamines were metabolized by O-methylation, deamination, glycol formation, oxidation, and conjugation. As a result of these findings, I then considered myself a neurochemist. This work also gave me a long-lasting interest in methylation reactions that I describe later. The metabolites of catecholamines, particularly MHPG, have been used as a marker in many studies in biological psychiatry.

A major problem in neurobiology research is the mechanism by which neurotransmitters are inactivated. At the time I described the metabolic pathway for catecholamines in 1957, it was believed that the actions of neurotransmitters were terminated by enzymatic transformation. Acetylcholine was already known to be rapidly inactivated by acetylcholinesterase. However, when the principal enzymes for the metabolism of catecholamines, catechol-O-methyltransferase and monoamine oxidase, were almost completely inhibited in vivo, the physiological actions of injected epinephrine were rapidly ended. These experiments indicated that there were other mechanisms for the rapid inactivation of catecholamines.

The answer to the question of the inactivation of catecholamines came in an unexpected way. When the metabolism of catecholamines was described, Seymour Kety and coworkers set out to examine whether or not there was an



abnormal metabolism of epinephrine in schizophrenic patients. To carry out this study, Kety asked the New England Nuclear Corporation to prepare tritium-labeled epinephrine and norepinephrine of high specific activity. The first batch of  $^3\text{H}$ -epinephrine that arrived in late 1957 was labeled on the 7 position, which we found to be stable. Kety was kind enough to give me some of the  $^3\text{H}$ -epinephrine for my studies. I thought it would be a good idea to examine the tissue distribution and half-life of  $^3\text{H}$  epinephrine in animals.

At about that time, Hans Weil-Malherbe spent three months in my laboratory as a visiting scientist, and together we developed methods for measuring  $^3\text{H}$ -epinephrine and its metabolites in tissues and plasma. To our surprise, when  $^3\text{H}$ -epinephrine was injected into cats, it persisted unchanged in the heart, spleen, and the salivary and adrenal glands long after its physiological effects were ended. This phenomenon puzzled us. We also found that  $^3\text{H}$ -epinephrine did not cross the blood-brain barrier. Just about this time Gordon Whitby, a graduate student from Cambridge University, came to our laboratory to do his PhD thesis. I suggested that he use methods for assaying  $^3\text{H}$ -norepinephrine similar to those we used for  $^3\text{H}$ -epinephrine to study its tissue distribution. As in the case of  $^3\text{H}$ -epinephrine,  $^3\text{H}$ -norepinephrine remained in organs rich in sympathetic nerves (heart, spleen, salivary gland). These studies gave us a clue regarding the inactivation of catecholamine neurotransmitters: uptake and retention in sympathetic nerves.

The crucial experiment that established that catecholamines were selectively taken up in sympathetic neurons was suggested by Georg Hertting from the University of Vienna, who joined my laboratory as a visiting scientist. In the next experiment, the superior cervical ganglia of cats were taken out on one side, resulting in a unilateral degeneration of the sympathetic nerves in the salivary gland and eye muscles. Upon the injection of  $^3\text{H}$ -norepinephrine, radioactive catecholamine accumulated on the innervated side, but very little appeared on the denervated side (13, 14). This simple experiment clearly showed that sympathetic nerves take up and store norepinephrine. In another series of experiments, Hertting and I found that injected  $^3\text{H}$ -norepinephrine taken up by sympathetic nerves was released when these nerves were stimulated (15). As a result of these experiments, we proposed that norepinephrine is rapidly inactivated by reuptake into sympathetic nerves. Other slower mechanisms for the inactivation of catecholamines proposed were removal by the blood stream, metabolism by O-methylation, and/or deamination at effector tissue or by liver and kidney.

In 1961, the first postdoctoral fellow, Lincoln Potter, joined my laboratory via the NIH Research Associates Program. The NIH Research Associate Program and the Pharmacology Research Associate Program provided an opportunity for recent PhD or MD graduates to spend two or three years in Bethesda doing full-time research. Because of the number of applicants for

this program, the investigators in the Intramural Program at the NIH would get the best and brightest postdoctoral fellows. During the past 25 years more than 60 postdoctoral fellows joined my laboratory to do full-time research. With one or two exceptions, most of the postdocs who worked in my laboratory went on to productive careers in research.

When a postdoc joins my laboratory I try to start him on a problem that has a good chance of success but is not trivial or pedestrian. There is an open and free exchange of ideas between my postdocs and myself, which makes it possible to try novel approaches to problems. By the time postdocs are ready to leave the laboratory, they are independent investigators. I found the interactions with bright and motivated young people stimulating and highly conducive to productive and original research.

When Linc Potter joined my laboratory, we directed our attention to the sites of the intraneural storage of norepinephrine. We suspected that  $^3\text{H}$ -norepinephrine, already shown to be taken up by sympathetic neurons, would label intracellular storage sites.  $^3\text{H}$ -norepinephrine was injected into rats, and their hearts were homogenized in isotonic sucrose; then the various subcellular fractions were separated in a continuous sucrose gradient. There was a sharp peak of radioactive norepinephrine in a fraction that coincided with endogenous catecholamines and dopamine  $\beta$ -hydroxylase, the enzyme that converts dopamine to norepinephrine. The norepinephrine-containing particles exerted a pressor response only when they were lysed. In another experiment,  $^3\text{H}$ -norepinephrine was injected, and the pineal gland, an organ rich in sympathetic nerve terminals, was subjected to radioautography and electron microscopy (16). Photographic grains of  $^3\text{H}$ -norepinephrine were highly localized over dense core-granulated vesicles of about 500 angstroms. All these experiments indicated that norepinephrine in sympathetic nerves was stored in small, dense core vesicles.

Subsequent studies with another postdoc, Dick Weinshilboum, showed that upon stimulation of the hypogastric nerve of the vas deferens, both norepinephrine and dopamine- $\beta$ -hydroxylase were discharged from the nerve terminals. This suggested that norepinephrine and dopamine- $\beta$ -hydroxylase were colocalized in the catecholamine storage vesicles of sympathetic nerves and were then discharged together by exocytosis (17). These findings led us to the postulation that the released dopamine- $\beta$ -hydroxylase would appear in the blood, which was soon confirmed. Later, our laboratory and others found abnormally low levels of plasma dopamine- $\beta$ -hydroxylase in familial dysautonomia and Down's syndrome, and high levels in patients with torsion dystonia, neuroblastoma, and certain forms of hypertension.

As soon as it was found that catecholamines could be taken up and inactivated by reuptake into sympathetic nerve terminals, I and my coworkers turned our attention to the effect of adrenergic drugs on this process. We



designed relatively simple experiments for this study, injecting the drug into rats and then measuring the uptake of injected  $^3\text{H}$ -norepinephrine in tissues. Cocaine was the first drug we examined. It had been postulated that cocaine causes supersensitivity to norepinephrine by interfering with its inactivation. After pretreatment of cats with cocaine, there was a marked reduction of  $^3\text{H}$ -norepinephrine in tissues that were innervated by sympathetic nerves after the injection of the radioactive catecholamine (18). This experiment indicated that cocaine blocked the reuptake of norepinephrine in nerves and thus allowed large amounts of the catecholamine to remain in the synaptic cleft and act on the postsynaptic receptors for longer periods of time. Using a similar approach, we observed that antidepressant drugs and amphetamine and other sympathomimetic amines also blocked the uptake of norepinephrine. In another type of experiment, using an isolated, perfused beating rat heart whose nerves had previously been labeled with  $^3\text{H}$ -norepinephrine, we found that the physiological action of sympathomimetic amines, such as tyramine, was mediated by releasing the norepinephrine from sympathetic nerves (19). After repeated treatment of the isolated heart with tyramine, the heart rate and amplitude of contraction were gradually reduced, presumably by the depletion of the releasable stores of the neurotransmitters. After replenishing the isolated heart with exogenous norepinephrine, the heart rate and amplitude of contraction of the isolated heart were restored. Amphetamine also released norepinephrine, and it was later shown by others that the physiological effects of the amine were due to the release of dopamine.

Most of my early work in catecholamines was done in the peripheral sympathetic nervous system. Hans Weil-Malherbe and I had found that catecholamines did not cross the blood-brain barrier. This made it impossible to study the metabolism, storage, and release of norepinephrine in the brain by peripheral administration of  $^3\text{H}$ -norepinephrine. It was Jacques Glowinski, a visiting scientist from France, who circumvented this problem. He devised a technique to introduce  $^3\text{H}$ -norepinephrine directly into the brain by injection into the lateral ventricle. Subsequent experiments showed that  $^3\text{H}$ -norepinephrine was mixed with the endogenous catecholamines in the brain. As in the peripheral nervous system, the  $^3\text{H}$ -norepinephrine was found to be metabolized by O-methylation and deamination. In a series of experiments we established that  $^3\text{H}$ -norepinephrine could serve as a useful tool in studying the activity of brain adrenergic nerves (13).

After labeling the brain adrenergic neurons, Glowinski and I examined the effect of psychoactive drugs on brain biogenic amines. We found that only the clinically effective antidepressant drugs block the reuptake of  $^3\text{H}$ -norepinephrine in adrenergic nerve terminals (20). This, together with the observation that monoamine oxidase inhibitors have antidepressant actions and that reserpine, a depleter of biogenic amines, sometimes causes depression, led to the formulation of the catecholamine hypothesis of depression

(21). We also found that amphetamines block the reuptake as well as the release of  $^3\text{H}$ -norepinephrine in the brain. Other investigators later showed that paranoid psychosis caused by excessive ingestion of amphetamines is due to the release of the catecholamine dopamine. One of the reasons that Les Iversen came to my lab as a postdoctoral fellow was to learn about the brain and its chemistry. Iversen and Glowinski worked extensively together in my laboratory on the effects of drugs on the adrenergic system in different areas of the brain. To conduct this study they devised a method of dissection of various parts of the brain that has become a classic procedure.

For several years our laboratory was concerned with adaptive mechanisms of the sympathoadrenal axis. One such mechanism, the induction of the catecholamine's biosynthetic enzyme, tyrosine hydroxylase, was observed in an unexpected manner, as often happens in research. Hans Thoenen, then working in Basel, asked to spend a sabbatical year in my laboratory. He and Tranzer had observed that injected 6-hydroxydopamine selectively destroys catecholamine-containing nerve terminals (22). I invited Thoenen to join my laboratory and bring 6-hydroxydopamine. The first experiment that Thoenen tried was to examine the effects of the destruction of peripheral sympathetic nerves on tyrosine hydroxylase. After the injection of 6-hydroxydopamine, as expected, tyrosine hydroxylase almost completely disappeared from sympathetically innervated nerves. A surprising observation was a marked elevation of tyrosine hydroxylase in the adrenal medulla. 6-Hydroxydopamine was known to cause persistent firing of nerves. We suspected that tyrosine hydroxylase was elevated in the adrenal medulla by continuous firing of the splanchnic nerve innervating the adrenals. This supposition was confirmed when other drugs that caused prolonged nerve firing, such as reserpine and  $\alpha$ -adrenergic blocking agents, also increased tyrosine hydroxylase (23). Subsequent experiments showed that increased nerve firing induced the synthesis of new tyrosine hydroxylase molecules in nerve cell bodies and the adrenal medulla in a transsynaptic manner. Similar results were obtained with another catecholamine biosynthetic enzyme, dopamine- $\beta$ -hydroxylase (13).

Another regulatory mechanism for catecholamine synthesis was found by asking the right questions rather than by serendipity. The ratio of epinephrine to norepinephrine in the adrenal medulla was known to be dependent on how much of the medulla was enveloped by the adrenal cortex. In species in which the cortex is separated from the medulla, norepinephrine is the predominant catecholamine, while in species in which the medulla is surrounded by the adrenal cortex, the methylated catecholamine, epinephrine, is by far the major amine. Dick Wurtman, a research associate in my laboratory, suggested an elegant experiment to determine the role of the adrenal cortex in regulating the synthesis of epinephrine. He removed the rat pituitary, a procedure that depleted glucocorticoid in the adrenal cortex, and then measured the effect on the levels of the epinephrine-forming enzyme, phenylethanolamine-N methyl-

transferase (PNMT), in the medulla. I had just characterized PNMT and found that it was highly localized in the adrenal medulla. The ablation of the pituitary caused a profound decrease in PNMT in the medulla after several days (24). The administration of ACTH, a peptide that increases the formation of glucocorticoids in the adrenal cortex, or the injection of the synthetic glucocorticoid, dexamethasone, increased PNMT in hypophysectomized rats almost to normal values.

## METHYLTRANSFERASE RESEARCH

After the description of catechol-O-methyltransferase, I became very much involved with methyltransferase enzymes (25). I spent most of my time at the lab bench working on methylating enzymes for many years. Soon after describing COMT, I turned my attention to the enzymatic N-methylation of histamine. A major pathway for histamine metabolism occurs via N-methylation. This prompted a search for a potential histamine-methylating enzyme. As in the case of other methyltransferases, I suspected that the most likely methyl donor would be *S*-adenosylmethionine. To make the identity of the histamine-methylating enzyme possible, Donald Brown, a postdoc in the lab of a colleague, and I synthesized [ $^{14}\text{C}$ -methyl]-*S*-adenosylmethionine enzymatically from rabbit liver with  $^{14}\text{C}$ -methylmethionine and ATP. Because of its ability to label the O or N groups of potential substrates by the transfer of  $^3\text{H}$ -methylmethionine, the availability of  $^{14}\text{C}$ -*S*-adenosylmethionine led to the discovery of a number of methyltransferase enzymes. Histamine N-methyltransferase was soon found and purified and its properties described. The enzyme is highly localized in the brain, and it also has an absolute specificity for histamine. Other methyltransferases soon discovered using [ $^{14}\text{C}$ -methyl]-*S*-adenosylmethionine were PNMT, hydroxyindole O-methyltransferase, the melatonin-forming enzyme, a protein carboxymethyltransferase, and a nonspecific N-methyltransferase. This latter enzyme was found to convert tryptamine, a compound normally present in the brain, to N-N-dimethyltryptamine, a psychotomimetic agent.

These methyltransferase enzymes, together with [ $^3\text{H}$ -methyl]-*S*-adenosylmethionine of high specific activity were used in developing very sensitive methods for the measurement of trace biogenic amines. We were able to detect, localize, and measure octopamine, tryptamine, phenylethylamine, phenylethanolamine, and tyramine in the brain and other tissues. The methyltransferases and [ $^3\text{H}$ -methyl]-*S*-adenosylmethionine also made it possible to measure norepinephrine, dopamine, histamine, and serotonin in 130 separate brain nuclei. Because of the sensitivity of the enzymatic micromethods, my colleagues and I were able to show the coexistence of several neurotransmitters in single identified neurones of *Aplysia* (26). Later, Thomas Hokfelt, using immunohistochemical techniques, demonstrated the coexistence of neurotransmitters in many nerve tracts (27).

## THE PINEAL GLAND

I was struck by an article from Aaron Lerner's laboratory, published in 1958, that described the isolation of 5-methoxy-N-acetyltryptamine (melatonin) from the bovine pineal gland, a compound that had powerful actions in blanching the skin of tadpoles (28). This compound attracted my attention for two reasons: it had a methoxy group and a serotonin nucleus. The methoxy group of melatonin had a special attraction for me. Also, at that time, serotonin was believed to be involved in psychoses because of its structural resemblance to LSD. I thought it would be fun to spend some time working on the pineal gland, an organ that was a mystery to me. The best way to start was to concentrate my efforts on aspects of the problem that I was familiar with, such as O-methylation.

Herbert Weissbach expressed an interest in collaborating with me in working out the biosynthetic pathway for melatonin. Weissbach had already made important contributions on the metabolism of serotonin. The availability of *S*-adenosyl-L-methionine with a radioactive methyl group provided an opportunity to examine whether the pineal gland could form labeled melatonin from potential precursor compounds. When we incubated bovine pineal extracts with N-acetylserotonin and [ $^{14}\text{C}$ -methyl]-*S*-adenosyl-L-methionine, a radioactive product that we soon identified as melatonin was found (29). Weissbach and I then purified the melatonin-forming enzyme, which we named hydroxyindole-O-methyltransferase (HIOMT), from the bovine pineal gland. We also found another enzyme that converted serotonin to N-acetylserotonin in the rat pineal. From these observations, we proposed that the synthesis of melatonin in the pineal proceeds as follows: tryptophan  $\rightarrow$  5-hydroxytryptophan  $\rightarrow$  serotonin  $\rightarrow$  N-acetylserotonin  $\rightarrow$  melatonin (30). Irwin Kopin, Weissbach, and I also found that melatonin was mainly metabolized by a microsomal enzyme via 6-hydroxylation. In a study of the tissue distribution of HIOMT we observed that the enzyme was highly localized in the pineal. This convinced me that the pineal was a biochemically active organ containing an unusual enzyme and product and was worth further study.

During 1960–1962 I spent little time doing pineal research. Most of my efforts were directed towards the biochemistry of catecholamines and the effect of psychoactive drugs. In 1962, when Wurtman joined my laboratory, I thought that he should devote most of his time to catecholamine research. As a medical student Wurtman had already made an important finding that bovine pineal extracts blocked gonadal growth in rats induced by light. Although pineal research was not a fashionable subject for research then, Wurtman and I were caught up by the romance of this organ, so we decided to spend our spare time working on the pineal. We thought that a good place to start was the isolation of the gonad-inhibitory factor of the pineal. Neither of

us wanted to go through a tiresome isolation and bioassay procedure, and we decided to take a chance and examine the effects of melatonin. We soon found that melatonin reduced ovarian weight and decreased the incidence of estrus in the rat (30).

Wurtman and I turned our attention to the effects of light on the biochemistry of the pineal. We found that keeping rats in the dark for a period of time increased HIOMT activity, compared to those kept in continuous light. This experiment gave Wurtman and me a biochemical marker to study how light transmits its message to an internal organ. Ariens Kappers had found that the pineal is innervated by sympathetic nerves arising from the superior cervical ganglia. This finding suggested an experiment to determine the effects of light on the pineal by removing the superior cervical ganglia and examining the effects of light and dark on the HIOMT. When the superior cervical ganglia were removed, the effects of light on HIOMT were abolished. This experiment told us that the effects of light on melatonin synthesis were mediated via sympathetic nerves arising from the superior cervical ganglia.

In 1964, Sol Snyder joined my laboratory as a postdoc, and he too was fascinated by pineal research. Quay had just made an important observation that the levels of serotonin, a precursor of melatonin in the pineal, are high during the day and low at night. Snyder and I developed a very sensitive assay for measuring serotonin in a single pineal. This gave us the opportunity to study how the serotonin rhythm, which can serve as a marker for the melatonin rhythm, is regulated by light in a tiny organ such as the pineal. We found that in normal rats in continuous darkness, or in blinded rats, the daily serotonin rhythm in the pineal persisted (31). This indicated that the indoleamine rhythms in the pineal were controlled by an internal clock. Keeping rats in constant light abolished the circadian serotonin rhythm, showing that light somehow stopped the biological clock. These experiments were the first demonstration that the rhythms of indoleamines in the pineal were endogenous and that they were synchronized by environmental lighting. We found that the circadian serotonin rhythm was abolished after ganglionectomy and also after decentralization of the superior cervical ganglion, indicating that the circadian clock for the serotonin and presumably the melatonin rhythm resided somewhere in the brain. Wurtman and I published an article in *Scientific American* in which we suggested that the pineal serves as a neuroendocrine transducer, converting light signals to hormone synthesis via the brain and noradrenergic nerves (32).

Shein, a psychiatrist at McLean Hospital, Wurtman, who was then at MIT, and I decided to see whether the rat pineal in organ culture metabolized tryptophan to melatonin, and it did. This finding provided an opportunity to examine whether the neurotransmitter of the sympathetic nerve, norepinephrine, could affect the synthesis of melatonin in pineal organ culture. The addition of norepinephrine to rat pineals in organ culture increased the

synthesis of melatonin from tryptophan. Shein and Wurtman then showed that noradrenaline specifically stimulated the  $\beta$ -adrenergic receptor.

For two years after 1970 I did little work on the pineal until Takeo Deguchi, a biochemist from Kyoto, joined my laboratory. Because interest in receptors was beginning to grow at that time, we decided that the pineal gland would be a good model to study the regulation of the  $\beta$ -adrenergic receptor. The activity of the  $\beta$ -receptor could be determined by measuring changes in serotonin N-acetyltransferase (NAT). David Klein previously showed that pineal serotonin N-acetyltransferase had a marked circadian rhythm that was controlled by a  $\beta$ -adrenergic receptor (33). Deguchi and I devised a rapid assay for N-acetyltransferase and soon confirmed Klein's findings. We then found that the nighttime rise in NAT was abolished by  $\beta$ -adrenergic blocking agents, reserpine, decentralization, ganglionectomy, and agents that inhibit protein synthesis (30). This told us that noradrenaline released from sympathetic nerves innervating the pineal gland stimulated the  $\beta$ -adrenergic receptor, which then activated the cellular machinery for the synthesis of NAT. Blocking the  $\beta$ -adrenergic receptor with propranolol at night or exposing rats to light also caused a rapid fall of NAT. These results indicated that unless the  $\beta$ -adrenergic receptor is stimulated by norepinephrine at a relatively high frequency, NAT rapidly decays. We thought that the rapid synthesis and fall of NAT would provide a useful model to study the molecular events in receptor linked synthesis of a specific protein (NAT) leading to the formation of a hormone (melatonin).

The regulation of supersensitivity and subsensitivity of receptors is an important biological problem. The rapidly changing pineal NAT provided a productive approach to study the mechanism of super- and subsensitivity of the  $\beta$ -adrenergic receptor (30). Procedures that depleted the neuronal input of noradrenaline in the rat pineal (denervation, constant light, or reserpine) caused a superinduction of NAT when rat pineals were cultured and treated with the  $\beta$ -adrenergic agonist, 1-isoproterenol. When pineal  $\beta$ -adrenergic receptors were repeatedly stimulated by injections of 1-isoproterenol into rats, the cultured pineals became almost unresponsive to the  $\beta$ -adrenergic agonist. In collaboration, my postdoctoral fellows Jorge Romero and Martin Zatz and I showed that the regulation of NAT and subsequent melatonin synthesis consists of a complex series of steps involving:  $\beta$ -adrenergic receptor, cyclic AMP, cyclic GMP, protein kinase, specific activation of mRNA for NAT, and synthesis of NAT (30). Decreased nerve activity induced by light caused an increase in receptor number and adenylate cyclase and kinase activity. This cascade of events then explained why a small change in release of noradrenaline from nerves causes a large change in pineal NAT. With the onset of darkness, there is an increase in sympathetic nerve activity that acts on the supersensitive receptor, cyclase, kinase, etc. This, we believe, considerably amplifies the signal (norepinephrine) to cause the large nighttime rise in NAT

formation. Klein later showed that norepinephrine acting on an  $\alpha_1$ -adrenergic receptor further amplified the NAT levels.

## THE LAST TEN YEARS

Because of space limitations I can only give a brief description of my research during the past ten years. Most of the research in my laboratory was concerned with how neurotransmitters transmit their specific messages. About ten years ago Fusao Hirata, a visiting scientist in my laboratory, and I observed that the occupation of certain receptors stimulated the methylation of phospholipids. On the basis of these findings we proposed a mechanism for the transduction of biological signals (34). This proposal generated considerable controversy, and the role of phospholipid methylation in signal transduction still remains to be resolved. Later, with the collaboration of several postdoctoral fellows, we reported on the interaction of stress hormones (catecholamines, ACTH, and glucocorticoids) and the multireceptor release of ACTH (35).

In 1984, I officially retired from government service at the age of 72. The NIMH allows me to keep my small laboratory and generously supports my research. With the help of postdoctoral fellows, I continue to be actively engaged in studying transduction mechanisms of neurotransmitters and hormones. We have evidence for a receptor-mediated release of arachidonic acid and its many metabolites via the activation of a GTP-binding protein linked to phospholipase  $A_2$  (36). This pathway promises to be an active area of future research.

F. Scott Fitzgerald once stated that there are no second acts in American lives. After a mediocre first act, my second act was a smash. So far the third act has not been so bad.

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